Malacological Applications of Scanning Electron Microscopy II. Radular Structure and Functioning

BY

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(6 Plates; 1 Text figure)

INTRODUCTION

This is the second report designed to introduce the capabilities of scanning electron microscopy to malacologists. Data on the basic advantages and operation of the scanning electron microscope (hereafter SEM) were presented in Part I (Solem, 1970), together with illustrations showing the kinds of information retrievable concerning shell surface features.

The SEM offers a 50× to 500× increase in depth of field and 12.5× to 100× increase in resolving power over optical equipment at equal magnification. Its range of magnifications, 14× to 100 000×, greatly exceeds optical ranges. When applied to any research problem, its immediate utility lies in providing illustration of small objects where depth of field problems have prevented adequate optical photography. Secondly, it permits seeing structures that were teasingly just at the limit of optical inspection, looking at them under higher magnification, then photographing these in essentially three-dimensional appearing fidelity. Finally, the ability to see and illustrate these small structures inevitably leads to investigations at a new level of inquiry. Questions concerning structures that were below the level of light microscopy can be asked and investigated. By use of ancillary analytic techniques, such as microprobe capabilities, a new world of investigations opens. Just as the classical microscopists of the early 1600's found an unknown world whose exploration has busied scientists for more than 300 years, so the new level of viewing introduced by the SEM will keep generations of scientists happily occupied.

The growing number of scanning electron microscopes has enabled several people to experiment with viewing radular denticles. Since the first report by Runham & Thornton (1967), papers by Thompson & Hinton (1968), Robertson (1970), Giusti (1970a, 1970b, 1970c), and Thomas (1971) include one to several SEM photographs. Generally the pictures were taken in direct vertical view of the teeth, much as in traditional optical examination of radular structure. Carriker (1969) studied the pattern of boreholes made by *Urosalpinx* in feeding, as well as functioning aspects of the muricid radula. Runham (1969) and Runham, Thornton, Shaw & Wayte (1969) utilized freeze drying and microprobe techniques to study radular function and mineralization. Undoubtedly additional papers will have been published by the time this report appears.

The present contribution is concerned with basic methods of specimen preparation and methods of viewing the material that increase the information retrieved. It also reports a previously unknown functional relationship between rows of teeth. Most of the work was done using a Cambridge Stereoscan instrument during the course of cooperative research with the American Dental Association Electron Optics Laboratory. I am indebted to Dr. Harvey Lyon, Assistant Secretary of the American Dental Association for enabling this work to be started, to Mr. John Lenke and Mr. George Najarian of the Electron Optics Laboratory for skilled tutoring in SEM techniques and machine operation, and to the American Philosophical Society for a grant that financed part of this work. The pictures in Plate 1 were made on a Jeolco SEM during a factory demonstration. Much of this work has developed from studies on the phylogeny of endodontoid land snails under sponsorship by National Science Foundation Grant GB-6779, whose assistance is gratefully acknowledged.

The technique outlined below has been found to give uniformly good results in handling pulmonate radulae. I have made very few mounts of prosobranch radulae, but Robert Robertson (personal communication) has had more success in his work on architectonicid radulae using

[1] Part of this material was presented at the 4th European Malacological Congress, Geneva, Switzerland, in September 1971.

different mounting substrates. The application of freeze drying or critical point drying probably will greatly reduce or eliminate membrane shrinkage, while continued experiments with coating and mounting media should refine and improve the quality of results to be obtained.

OBJECTIVES IN SPECIMEN PREPARATION AND VIEWING

Traditional optical study of radulae involves maceration, cleaning, and mounting the radula in a squashed position between two pieces of glass. The literature on these procedures is extensive, but an excellent and convenient summary is given by MEEUSE (1950). Viewing of the teeth is, by necessity, restricted to one angle, essentially a perpendicular axis looking down onto the cusps. The teeth themselves are in a flattened position, lying folded together as normally occurs at the posterior part of the buccal cavity. Adopting this angle for SEM viewing negates the main advantage of the instrument, its great depth of field, and continues to hide extremely important aspects of both tooth structure and function. The most important thing to learn is that by utilizing the extreme tilts and rotational flexibility of the SEM to view the teeth from a variety of angles, the information content of observations will be improved several times over. It must be remembered that the teeth lie flat and folded only when not being used. During feeding the midanterior part of the radula is curved and the teeth partly to strongly erected (MARKEL, 1957, figure 10). Normal curvature of the anterior radular section shows the teeth in erection. This can be studied by histological techniques (see MARKEL, *op. cit.*). However, during actual feeding the individual teeth are subject to great stress and pressure as they slice into the object being eaten. The report below of a sophisticated pattern of interrow support for the teeth during the stress of a feeding stroke is a significant addition to our knowledge of radular functioning.

Obtaining these types of information requires unlearning habits of radular preparation. The flattened radula for optical viewing gives only one part of the radular pattern. By deliberately making folds and bends in the radula (Figure 1) it is possible to simulate a feeding stroke on one section, then to show the folded position on another part. If longitudinal tears are made in a few places, then detailed side views of individual teeth become possible. If a radula is folded or twisted over diagonally, then information on basal supports and feeding stroke position can be gained simultaneously. The more bends, folds and tears, the greater the variety of information that can be extracted from a single mount. While the carefully flattened, intact radula allows counts of rows and tooth numbers, it fails to yield much information on individual tooth structure and form. A torn, undulating radular mount with several creases and folds is far preferable for SEM examination.

Equally important is a knowledge of the special problems involved in SEM viewing of biological objects. First of these is the necessity for very careful cleaning of the specimen. At the magnifications used, 100–30 000×, a bit of lint becomes a cable, a speck of dirt a boulder.

Second is the necessity to discharge current from the specimen. Pulmonate radulae are non-conductive of elec-

Explanation of Figures *1* to *5*

Subulina octogona (Bruguière, 1792)

Figures *1 - 5*: Lower slopes of Mt. Sauniatu, 200 - 300 feet elevation, Upolu, Western Samoa. Field Museum of Natural History no. 152859. Figure *1*: unworn lateral teeth in late feeding stroke position at 2 075 ×. Figure *2*: worn lateral teeth from anterior end of radula at 2 050 ×. Figure *3*: unworn mid-marginal teeth at 2 100 ×. Figure *4*: worn early marginal teeth at 2 075 ×. Figure *5*: unworn outermost marginal teeth at 2 100 ×.

Explanation of Figures *6* to *11*

Suteria ide (Grey, 1850)

Figures *6, 7*: Waiwera-Pohoi Road, north of Auckland, North Island, New Zealand. Field Museum of Natural History number 135430. Figure *6*: unworn lateral teeth from posterior end of radula at 2 425 ×. Figure *7*: worn lateral tooth from next to front row at anterior end of radula at 4 250 ×.

Ptychorhytida aulacospira (Pfeiffer, 1846)

Figures *8 - 10*: Summit of Col de Momen, near Moindou, West central coast of New Caledonia. Field Museum of Natural History no. 159331. Figure *8*: near vertical view of two lateral teeth at 160 ×. Figure *9*: near horizontal view from left side of tooth at 460 ×. Figure *10*: view from 45° angle of right side of tooth at 265 ×.

Pleuropoma sublaevigata (Pfeiffer, 1852)

Figure *11*: Santo, Espiritu Santo, New Hebrides. Field Museum of Natural History no. 109460. Teeth at 805 × magnification in elevated position, from bottom to top of photograph – R - central; the slender and elongate tricuspid A - central and B - central; a spoon-shaped C - central hidden under the edge and acting as support to the huge capituliform complex; the seven-cusped comb-lateral; and in the upper right margin a few marginal teeth visible.


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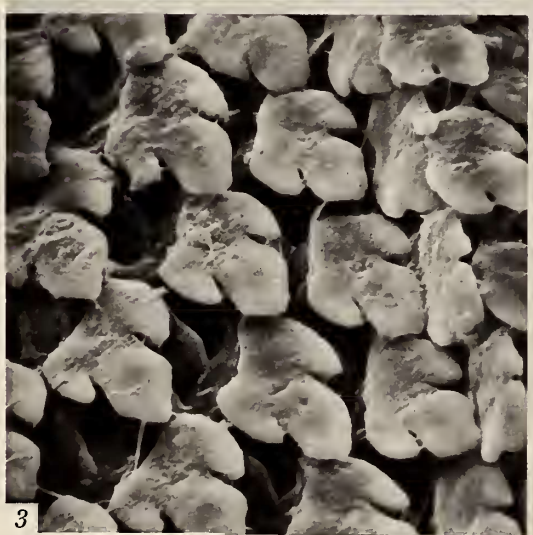
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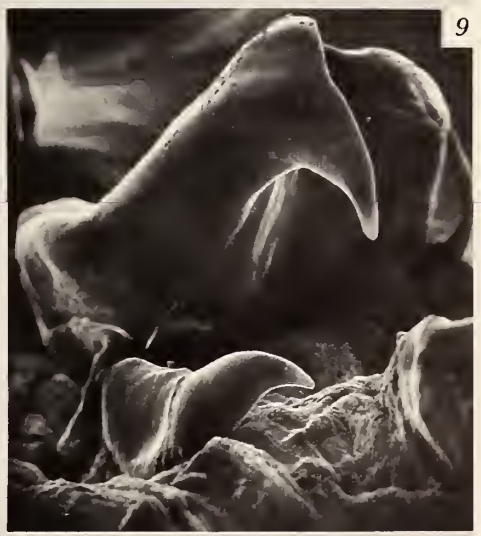
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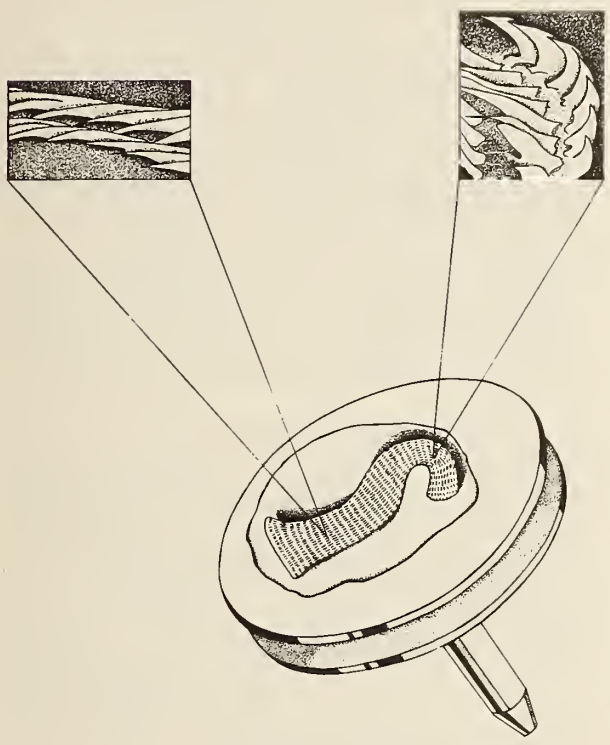

Figure 1

SEM stub showing pattern of radular mount to enable viewing of teeth in folded (left) and erected (right) position

tric current. If the current is not bled off but allowed to accumulate, then the phenomenon called "charging" occurs and the screen image dissolves into virtually a single bright spot. "Charging" will occur either if the lateral surfaces of the individual teeth are not coated with a metallic film, or if sufficient portions of the radular membrane are not in contact with the substrate. There must be a continuous metallic coating leading from the radular surface first to the mounting substrate and then to the specimen stub for proper discharging of the current. If, for example, the radula is put onto double masking tape or laid upon a film of silver paint, it will dry with edges and parts of the central portion curled up from the surface. This can easily reduce the total contact area of the radula to a point that, regardless of how good a coating job is done, the ratio of contact to noncontact area is below the level needed for adequate bleeding off of the current.

Successful SEM viewing of radulae thus requires abandonment of traditional flat mounting and perpendicular viewing, careful cleaning of the specimen, mounting with deliberate distortion on a substrate that will provide adequate surface contact area to permit effective discharge of the current, and coating with a metallic film on the sides as well as the top of the individual teeth.

SPECIMEN PREPARATION

While it is possible to dissect the radula from the buccal mass, remnant tissues will obscure much of the posterior portion. With smaller sized specimens, dissection becomes increasingly difficult. For general viewing, it is far simpler and more satisfactory to macerate the entire buccal mass in potassium hydroxide (KOH). A 1½ inch Stender dish is filled half full of distilled water and 12–13 KOH pellets are added. Tap water is not used since it may contain mineral impurities that can settle out on the surface of the teeth. After the pellets have dissolved, the buccal mass is immersed in the solution and allowed to sit until the muscle tissue falls apart at being touched, but not until it is a dissolved "soup." This will take from three to seven hours, depending on the size of the buccal mass, the preservative in which it has been stored, and whether the snail was a herbivore or carnivore. Temperature of the solution is not critical, with good results having been obtained at room temperature extremes of 60° F and 97° F (15½° and 36° C). Specimens that have been preserved in formalin require longer to macerate than do alcohol preserved materials, while freshly killed specimens require the shortest time. Buccal masses of herbivorous snails macerate readily, but in many carnivores the heavy muscle layers can only be removed by use of an ultrasonic cleaner after the buccal mass has been left for 12–24 hours in the KOH solution. To minimize airborne particle contamination of the tooth surfaces, the Stender dish is covered during the maceration process.

Under a dissecting microscope, the radula is teased loose from the disintegrating muscles, adherent membranes are pulled off, and then the radula transferred into a small vial half filled with 70% ethyl alcohol. Depending on the size of the radula, a 5 × 20 mm to a 10 × 40 mm vial is used. The vial is immersed part way into the tank of an ultrasonic cleaner for 5–15 seconds. This serves to shake loose from the radular surface any film of KOH, virtually all particles of macerated tissue, and to grossly dilute any colloidal suspension of macerated tissue. If the alcohol in the vial becomes noticeably "cloudy" with suspended matter, then the radula is washed for a few minutes in another Stender dish filled with 70% ethyl alcohol.

After this, it is treated to another immersion in the ultrasonic cleaner. Finally, the cleaned radula is allowed to sit overnight in another wash of 70% alcohol. If that solution shows precipitated materials, then one or two more alcohol washes or sonic cleaner treatments, or both, are given the radula. The importance of this cleaning procedure cannot be overstressed. In early preparations, such as that of *Subulina octona* (Figures *3, 5*), the ultrasonic immersion was omitted. The fine particles on the teeth are recrystallized KOH, while the partly dissolved membranous coating is muscle tissue. The lack of such extraneous matter in other specimens (Plates 2–6) indicates the utility of this technique.

Minuten insect pins mounted in wooden match sticks provide effective tools for manipulating the radula. A few holes and tears are made in its surface and the radula is partly unfolded and creased. Brief washes in 95% alcohol and absolute alcohol can be helpful in slightly reducing shrinkage of the basal membrane, but equally good mounts have been obtained by working directly from 70% alcohol. Equipment to enable trying critical point drying or standard freeze-drying techniques has not been available to me, but experiments with these procedures should yield worthwhile results.

SPECIMEN MOUNTING ON SEM STUB

The objectives in selecting a mounting medium are to insure a firm bond between both the edges and mid-section of the radula with the medium itself, to have a surface that will dry with contours in it to maximize the degree of folding and creasing in the radula, and to avoid the radula sinking into and being covered by the medium during the mounting process. Excellent results have been achieved using Sanford's Rubber Cement, with much poorer results from Duco cement, Elmer's Glue or similar substances. The rubber cement is viscous enough that the radula will sit on its surface rather than having some of the mounting material flow up and over the sides of the radula as usually happened with the other compounds.

Actual mounting procedures are as follows. First, an SEM stub is cleaned with alcohol and wiped with a lint-free "Kimwipes" tissue. Use of a cloth or ordinary paper toweling usually deposits micro-lint particles on the stub surface. A mound of plasticine is placed on a glass slide and the shaft of the SEM stub (Figure 1) pressed into the plasticine. This permits easy shifting of the mount under the dissecting microscope. The surface of the stub should be slightly angled from the horizontal. A straightened paper clip makes a handy tool for transferring rubber cement to the stub.

The slide and the angled stub are placed under a dissecting microscope at low power. Using a paper clip, a dab of rubber cement that is slightly larger than the area of the radula to be mounted is made on the stub. This is immediately covered with several drops of the same alcohol concentration that the radula is in. If air bubbles are trapped in the rubber cement, there will be greater contours. A little practice in making dots, streaks or large blobs of rubber cement will enable preparing an effective mount for any particular radula. If more than one radula is to be mounted on a stub for viewing, then the dot should be made on the low part of the angled stub. With practice, the alcohol drop can thus be kept to one-third or one-half of the stub and hence not cover a previously mounted radula. The function of the covering drop of alcohol is to prevent the rubber cement from drying until after the radula has been placed in position successfully. The alcohol solution can be renewed as needed. It will

Explanation of Figures *12* to *17*

Papuina phaeostoma medinensis I. Rensch, 1934

Figures *12 - 16*: Lossu Village, 80 miles southeast of Kavieng, New Ireland, Bismarck Archipelago. Field Museum of Natural History no. 168394. Figure *12*: unworn central and first lateral teeth at 840 ×. Figure *13*: marginal teeth showing twisted nature of supporting structure at 600 ×. Figure *14*: mid-lateral teeth showing shape of basal support structure at 990×. Figure *15*: unworn lateral teeth viewed from a low angle looking backward along the rows of teeth at 745 ×. Figure *16*: worn lateral teeth from anterior end of radula viewed from same angle as in Figure *15* at 850 ×.

Parvatella flemingi (Pfeiffer, 1857)

Figure *17*: Seven miles west of Jalalabad, Nangarhar Province, Afghanistan. Field Museum of Natural History no. 147172. Single marginal tooth from right side of radula at 1 560 ×.

Explanation of Figures *18* to *20*

Achatinella bellula E. A. Smith, 1873

Figures *18 - 20*: Windward side of Koolau Crest above Wilson Tunnel, 1 400 - 1 600 feet elevation, Kalihi Valley, Oahu, Hawaii. Field Museum of Natural History no. 167977. Figure *18*: pattern of cusp overlap and details of cusp structure at 3 664 ×. Figure *19*: displaced tooth showing size of basal plate and pattern of interrow overlap at 1 880 ×. Figure *20*: vertical view of cusp structure at 2 340 ×.


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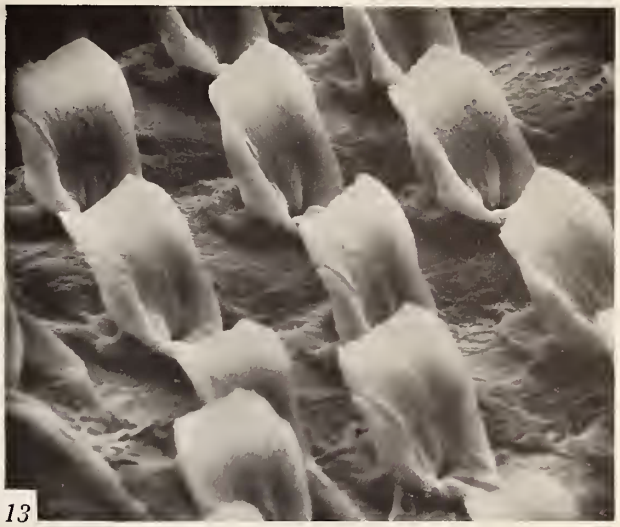
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